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# 博 士 論 文 概 要

## 論 文 題 目

*In vitro* screening of peptides and proteins by DNA display.

DNA ディスプレイ法によるペプチド及び
タンパク質の試験管内スクリーニング

申 請 者

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Genome projects have revealed genome sequences of many organisms including the human genome. Genomes contain many protein-coding genes and human is thought to have 30,000-40,000 protein-coding genes. However, functions of many genes are unknown and we are still far from understanding complicated biological phenomena. Therefore, it is important to clarify functions of proteins. One way to analyze the function of a protein is to search its interaction partner, *i.e.*, to analyze protein-protein and small molecule-protein interactions.

In the 1st chapter, I give an overview of current technologies to detect protein-protein and small molecule-protein interactions and mention the merits and demerits of each technology. I also refer to the significance of novel technologies based on *in vitro* translation that permits totally *in vitro* screening of protein-protein and small molecule-protein interactions. Further, I explain the concept of a novel technology designated “DNA display” and the aim of this study, *i.e.*, to establish the DNA display system for proteome research.

In the 2nd chapter, I report optimization of DNA constructs to achieve efficient formation of “DNA-protein conjugates”. DNA display employs water-in-oil emulsions formed with *in vitro* transcription/translation system and mineral oil to generate DNA-protein conjugates in which DNAs (genotype) are linked to their corresponding proteins (phenotype). These DNA-protein conjugates are subjected to affinity selection followed by identification by sequencing. Therefore, formation of DNA-protein conjugates is a crucial step in DNA display. First, I combined various promoters, translational enhancers at the 5’-untranslated region and coupled *in vitro* transcription/translation systems, and examined efficiency of protein synthesis. A combination of SP6 promoter and a translational enhancer derived from tobacco mosaic virus dramatically accelerated protein synthesis in a wheat germ extract transcription/translation system. By employing this combination, I achieved highly efficient (more than 95%) formation of DNA-protein conjugates.

In the 3rd chapter, I describe the preparation of water-in-oil emulsions using wheat germ extracts. The concept of DNA display requires uniform emulsions so that most of DNA molecules can be separately compartmentalized in each micelle. By measuring the size distribution of droplet diameter of emulsions by laser diffraction, I found that appropriate emulsions can be formed with wheat germ

extracts. Judging from the average diameter, I conclude that more than $10^9$ DNA molecules can be subjected for selection.

In the 4th chapter, I optimized selection conditions in a model system using FLAG peptide and anti-FLAG M2 antibody. By optimizing binding, washing and elution conditions, I successfully enriched the desired gene by a factor of 100-fold per selection round. I also show that desired gene can be enriched by conducting multiple rounds of selection even when it was mixed to undesired genes at the ratio of 1:100,000.

In the 5th chapter, I describe a trial to enrich peptides from a completely random sequence library, which in turn demonstrates the applicability of DNA display. A random decapeptide library was constructed using a long degenerate oligonucleotide primer and subjected to screening of peptides that bind to anti-FLAG M2 antibody for five selection rounds. By analyzing enriched DNA by immunoblotting, I found that the fraction of peptides capable of binding to the anti-FLAG M2 antibody clearly increased at round 4 and saturated round 5. Consistently, protein microarray analysis of randomly chosen clones from rounds 3 to 5 showed that most peptides from round 5 bound to the antibody. Sequence analysis of enriched clones revealed that 71 enriched clones were grouped into 21 distinct FLAG-like sequences. Amino acid residues D, Y, K and D in the 1st, 2nd, 3rd and 6th positions of the original FLAG sequence (DYKDDDDK), which are known to be critical to binding to the anti-FLAG M2 antibody, were conserved at high frequencies. I also investigated binding affinities of selected peptides by pull-down assays and surface plasmon resonance (SPR) spectroscopy. A few of the selected peptides showed significantly higher affinities than that of the original FLAG peptide.

In the 6th chapter, I describe applications of DNA-display to folded proteins. When proteins are fused to streptavidin (SA), formation of DNA-protein conjugates was moderately inefficient in some proteins tested. I overcame this problem by introducing peptide linkers between SA and the fused proteins, resulting in highly efficient formation of DNA-protein conjugates in any protein tested including a 61-kDa protein, luciferase. I conclude that efficient tetramrerization of SA fusion proteins is conferred by separating the two domains and that this in turn enables efficient formation of DNA-protein conjugates.

As model experiments, Glutathione S-transferase (GST), FKBP12 (FK506 binding protein 12 kDa), HP1β (heterochromatin protein 1β) and a Polycomb-group protein RYBP (RING1A-and-YY1 binding protein) were selected on matrices coupled with corresponding bait (glutathione, FK506, histone H3 N-terminal peptide methylated at Lys9 and RING1A, respectively). While specific enrichment of the desired genes was successful when DNA-protein conjugates were prepared separately in non-emulsified reactions containing high concentration (10 nM) of DNA, it was severely reduced or even undetected when DNA-protein conjugates were prepared in standard emulsified reactions where DNA concentration was adjusted to 25-100 pM. In this situation, difference between specific and non-specific binding of DNA-protein conjugates to the bait-coupled matrix would be trivial, resulting in reduction or even loss of enrichment. Therefore, I conclude that it would be necessary to prepare enormous amount and highly concentrated prey and/or to reduce non-specific binding by using effective blocking reagents for further improvements.

In the 7th chapter, I give conclusions to this study. From the aspect of screening of peptide ligands, I made an epoch-making achievement. DNA display excels in rapidity and simplicity compared with any other technology for screening peptide ligands reported to date such as phage display, and would be ideal for this purpose. However, enrichment of folded proteins from large libraries has been very inefficient or unsuccessful. Taking into account both merits and drawbacks of DNA display, some of which have emerged through this study, I discuss potential of DNA display and give future perspectives.